Stem Cell Biology and Regenerative Medicine

Maria Gazouli
George E. Theodoropoulos *Editors*

Digestive System Diseases

Stem Cell Mechanisms and Therapies

Humana Press

Stem Cell Biology and Regenerative Medicine

Series Editor:

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Our understanding of stem cells has grown rapidly over the last decade. While the apparently tremendous therapeutic potential of stem cells has not yet been realized, their routine use in regeneration and restoration of tissue and organ function is greatly anticipated. To this end, many investigators continue to push the boundaries in areas such as the reprogramming, the stem cell niche, nanotechnology, biomimetics and 3D bioprinting, to name just a few. The objective of the volumes in the Stem Cell Biology and Regenerative Medicine series is to capture and consolidate these developments in a timely way. Each volume is thought-provoking in identifying problems, offering solutions, and providing ideas to excite further innovation in the stem cell and regenerative medicine fields.

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ISSN 2196-8985 ISSN 2196-8993 (electronic)
Stem Cell Biology and Regenerative Medicine
ISBN 978-3-030-11964-5 ISBN 978-3-030-11965-2 (eBook)
https://doi.org/10.1007/978-3-030-11965-2

Library of Congress Control Number: 2019935572

This Humana Press imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

This book is dedicated to our families

Preface

Digestive System Diseases: Stem Cell Mechanisms and Therapies

The gastrointestinal tract has a rapid epithelial cell turnover, which remains throughout life. Stem cells play a key role in the regulation and maintenance of this process and give rise to all the gastrointestinal epithelial cell lineages. The identification of specific markers for the gastrointestinal stem cells, along with the technological advantages to track their endogenous activity and to exploit their ability to generate new epithelia, has significantly improved our understanding of stem cell-driven homeostasis and pathogenesis of gastrointestinal diseases. These exciting new insights in the implication of stem cells into the gastrointestinal system pathologies might lead to the potential development of stem cell-based therapies.

This book places the current developments in the gastrointestinal stem cell field clearly in context. It will hopefully serve as a useful tool, concentrating current knowledge on this "hot" topic, which has been currently attracting researchers' and clinicians' interest. Additionally, this book is a referral textbook for whoever would like to enhance his/her knowledge on stem cells.

The authors focused on digestive diseases and analyzing stem cell contribution on each of the digestive system's parts. Whether you are a student, researcher, clinician, or patient, or just interested in digestive diseases, we hope you enjoy this book as much as we have enjoyed researching and organizing it!

Athens, Greece

Maria Gazouli
George E. Theodoropoulos

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About the Editors

Maria Gazouli, PhD, Biologist is Associate Professor of the Department of Biology at the National and Kapodistrian University of Athens (NKUA), School of Medicine. She performed her first PhD training in the Department of Biology, School of Science of NKUA in partnership with the Hellenic Pasteur Institute; her second PhD training in the Department of Microbiology, School of Medicine of NKUA; and her postdoctoral work in the USA (1997–2000) in the Cell Biology and Pharmacology Department, Georgetown University Medical Center, Washington, DC. In 2007, she joined the NKUA Medical School as Lecturer of Biology; in 2012, she was promoted to Assistant Professor of Molecular Biology; and in late 2016, she was promoted to Associate Professor of Molecular Biology. Dr. M. Gazouli's work refers mainly to the molecular basis of diseases (mainly autoimmune diseases and cancer), to molecular detection of pathogens, and to the investigation of the pathogenesis of the diseases they cause in humans. She worked on stem cell implication in mucosal healing and is responsible for the e-learning program of the NKUA Stem Cells and Regenerative Medicine. Recently, Dr. Gazouli was involved in the incorporation of nanotechnology into targeted cancer detection, imaging, and drug delivery. She was honored with a Fulbright Scholarship for the Development of Nanotechnology-Based Biosensor Arrays for the Detection of Circulating Colorectal Cancer Cells at the University of Maryland, College Park, MD, USA. Dr. Gazouli's work is reflected in more than 230 publications that have received more than 8900 citations and

an h-index of 48 (Google scholar, 1/10/2018). She owns one granted international patent and three European patents. She has given more than 50 invited lectures at international and national conferences and universities and has trained several junior scientists. She has served as ad hoc reviewer for various high-impact scientific journals and is regularly invited to serve on review panels as an expert evaluator by prestigious organizations, such as the National Research Grant Funding Agencies of Greece; Broad Medical Research Program Inflammatory Bowel Disease Grants; the National Science Centre (Narodowe Centrum Nauki), Krakow; the PISCOPIA Fellowship Programme on behalf of the University of Padova, Italy; the Czech-Norwegian Research Programme; the Qatar National Research Fund; the Danish Council for Independent Research for DFF, YDUN Research Project; the Italian Ministry of Education, Universities and Research (MIUR); and the evaluation of research products.

George E. Theodoropoulos was born in Greece in 1969 and graduated from Athens Medical School in 1992. His PhD research in Tumor Markers of Gastrointestinal Malignancies was completed in 1994. He completed a 6-year residency program in General Surgery and a fellowship in Colon and Rectal Surgery in the USA. Following a 4-year course as a Private Surgeon in Athens Medical Center, he was elected as a Lecturer of Surgery in Athens Medical School in 2007. He is currently holding an academic post as an Associate Professor of Surgery at the same university. He is a Diplomat of the American Board of Surgery and of the American Board of Colon and Rectal Surgery and a Fellow of the American College of Surgeons (FACS) and of the American Society of Colon and Rectal Surgeons. Twenty PhD theses have been completed under his supervision. He has also completed a 6-month research fellowship in the Department of Colorectal Surgery, Cleveland Clinic Florida, Weston, FL, USA. He has set up and guided the function of a clinic of surveillance of health-related quality of life and oncologic process of postoperative colorectal cancer patients; has been supervising the Colorectal Unit of

the First Department of Propaedeutic Surgery of Athens Medical School at Hippokration Hospital, Athens, Greece; and has recently established and has been coordinating, along with the Radiology and the Academic Gastroenterology Department of the hospital, a "Lower Digestive Tract Study Unit," aiming at the multidisciplinary approach of large bowel diseases. He has presented at more than 200 national and international meetings and invited talks at 90 meetings. He is the author/coauthor of 120 internationally cited, peer-reviewed journal publications. His research work has international recognition, and there are more than 4,000 citations of his publications related to his research (h-index 34).

Introduction: Gastrointestinal Stem Cells in Human Health and Disease

Maria Gazouli and George E. Theodoropoulos

Histologically, the human gastrointestinal tract is composed of a series of epithelial cells (ECs) highly compartmentalized in terms of morphology and function. These epithelia are renewed on a periodic basis for the homeostasis to be preserved. Regeneration can also occur following tissue damage so as for the tissue integrity and compartmentalization to be retained. However, there are conditions that can lead to the formation of lesions, including metaplasia and dysplasia. The former refers to the replacement of one differentiated cell type by another type of differentiated cell; these lesions are related to a high risk of intestinal cancer [1]. Dysplasia refers to an abnormality of development, growth, or differentiation. Since intestinal epithelial cells are renewed by local intestinal stem cells (ISCs) and the regulation of their functions, most importantly proliferation and differentiation, is associated with these lesions, the regulatory mechanisms related to these cells need to be enlightened.

Recently, the identification of specific markers for ISCs resulted in a better understanding of the regulation of homeostasis and regeneration of the small intestine. Its histological structure includes a mono-stratified epithelium that forms two anatomical structures: the crypts and the finger-like protrusions known as villi in which different cellular types can be found. The crypts harbor stem cells and Paneth cells and transit amplifying cells, and the villi harbor ECs, goblet cells, and enteroendocrine cells (EEs). Paneth cells are located at the crypt's base, closely associated with stem cells and secreting antimicrobial substances and lysozyme. Stem cells

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M. Gazouli, G. E. Theodoropoulos (eds.), *Digestive System Diseases*, Stem Cell Biology and Regenerative Medicine, https://doi.org/10.1007/978-3-030-11965-2_1

serve a dual purpose: self-renewal and generation of TA cells which will generate all the differentiated cell types of the villi, maintaining the epithelial homeostasis. The newly identified markers can divide these stem cells into two subpopulations: the crypt base columnar stem cells (CBC) which as their name suggests lie on the base crypt and the +4 cells which can be found four cellular diameters apical of the crypt's base. CBC cells express the Leu-rich repeat-containing G protein-coupled receptor 5 (LRG5) which is a gene targeted by Wnt. Follicle epithelial stem cells are also labeled by Wnt, a fact that suggests that LGR5 could serve as a marker for Wnt-activated stem cells [2]. Epithelial cells from the base of colonic crypts have been cultivated in vitro and behave as multipotent stem cells [3], while Lgr5-GFP positive cells may be able to generate uniform intestinal organoids which is a characteristic ISC property [4].

The esophagus, the tube that connects the throat to the stomach, consists of a stratified epithelium without the distinct structures of the small intestine (including the crypts) and multiple layers of squamous keratinocytes. Here, the proliferating cells are the basal cells which are attached to the basal membrane. From there, the cells migrate to the upper layers and eventually shed inside the lumen. However, it remains unclear whether all the basal cells or just a subpopulation have stem cell characteristics; the results of the available studies are rather conflicting [5–9].

The human stomach consists of three regions: the cardiac, the corpus, and the pylorus. The corpus is composed of an epithelium with gastric units and structures that resemble the small intestine's crypts and project deep inside the mucosa. The four different cell types that can be found lead to the subdivision into four regions. The pit region which is close to the lumen contains mucous cells. Beneath these, the isthmus harbors stem cells which proliferate rapidly. Below the isthmus, the neck region can be found, in which gland mucous cells are contained. In its base, there the last category of cells, the chief cells, is responsible for the secretion of several digestive enzymes. Parietal cells which produce acid can be found in all these regions. The first stem cells to be recognized are in the isthmus zone; these stem cells can regenerate all the differentiated cell types. Another rare stem cell population has been tracked along the gland in the pylorus. Typically, these cells are dormant, but during injury they can regenerate all different cellular types. Studies have revealed more stem cell sites (in the pylorus and corpus near the isthmus and at the bottom of the gastric unit in the corpus), a fact that suggests the gastric's epithelium plasticity.

In the human small intestine, ISC functions are fine-tuned by a plethora of factors that derive from the stem cell niche. This formation comprises adjacent epithelial cells, myofibroblasts, neurons, lymphocytes, and the basal membrane. Of note is that the Wnt activity shows different activity inside the crypt with the most increased at the crypt's base. This gene is vital for ISC proliferation and determines cell fates within the crypt; if the Wnt signaling is lost, the intestinal crypts are ablated.

Another important regulatory mechanism is the Bone Morphogenetic Protein (BMP) signaling which exhibits its highest activity toward the villus and its lowest at the crypt's base. Depletion of its receptor 1a leads to opposite effects from those

that occur when Wnt signaling is lost. Progenitor and proliferative stem cells expand, and the inhibition of BMP signaling leads to the formation of ectopic crypts. This indicates that ISC proliferation is negatively regulated by the BMP signaling. These signaling molecules are affected by Hedgehog (Hh) signaling which increases BMP signaling and reduces Wnt signaling activity; epithelial precursor cells are reduced. The inhibition of Hh signaling leads to defective villus formation and results in a hyperproliferative epithelium [10].

As knowledge around the regulation of gastrointestinal stem cells evolves, the origin and progress of epithelial lesions become clearer. Metaplasia typically occurs in epithelial tissues exposed to the environment (esophagus and stomach) [1]. The most common metaplasias reported in humans are Barrett's esophagus and intestinal metaplasia (affecting the gastric region). Normally, the esophagus is lined by multiple squamous cell layers which during this condition are replaced with cells that form an intestine-like columnar epithelium. This disease is an important risk factor for esophageal adenocarcinoma. Treating options include the inhibition of acid production, anti-reflux surgery, chemoprevention, and ablation therapy. Several studies have been made to clarify the mechanism of the disease with some of them suggesting that either this condition involves stem cells from the cardiac region of the stomach or that the normal esophageal stem cells change their identity leading to the disease.

Another type of metaplasia, the intestinal metaplasia, occurs when intestinal epithelial cells are present in the stomach. This condition includes two stages: the complete intestinal metaplasia which occurs in the early phase (the metaplastic epithelium is like the mucosa of the small intestine and has ECs and goblet cells) and the incomplete intestinal metaplasia which occurs in later stages (the metaplastic epithelium resembles the morphology of the large intestine and includes only goblet cells). Both stages express an intestinal-specific marker mucin 2 (MUC2), and at the same time, the gastric-specific marker mucin 6 (MUC6) is lost. Furthermore, intestinal metaplasia may result in gastric dysplasia, a precancerous state that can lead to gastric cancer.

Helicobacter pylori infection, a major cause of gastric cancer, is believed to enhance cellular proliferation [11] and to negatively affect the maturation of precursor cells. Bone marrow-derived cells transform into metaplastic cells acting as a source of intestinal metaplasia and possibly gastric cancer, but the mechanism remains unclear [12].

Dysplasia is a common finding at neoplastic stages, and the progression to an invasive cancer phenotype is rapid both in the stomach and the colon [13–15]. The initialization of the invasive gastric cancer occurs when dysplastic cells cross the barrier of the basal membrane. Histologically, based on the degree of the cellular abnormality, dysplasia can be characterized as either high- or low-grade dysplasia. Precancerous metaplastic sites can transit to dysplasia with a varying progression rate. Inflammatory bowel disease (IBD) which includes ulcerative colitis and Crohn's disease is associated with colorectal cancer. Inflammation boosts cancer progression though the secretion of growth and survival factors which limit apoptosis

and increase cell proliferation. Additionally, inflammation cells release reactive oxygen species which disturb genome integrity [16, 17].

Of notice is that both lesions (metaplasia and dysplasia) require the transformation or trans-differentiation of epithelial cells; these events most likely involve changes in the transcriptome which is responsible for the cell's identity. Several transcription factors have been related to these transitions including the homeodomain transcription factors Cdx1 and Cdx2 which define prospective intestinal cells but not cells from the gastric region. Metaplasia has been also associated with ectopic expression of CDX genes which eventually forms intestinal tissues in the stomach [18–20]. Furthermore, the development of these lesions is linked to the deregulation of gastrointestinal stem cells, and thus a better understanding of their regulatory mechanisms (which include cellular identity) is vital for these pathologies to be treated.

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Introduction to Stem Cell Principles and Biology

Maria G. Roubelakis

Embryonic Stem Cells (ESCs)

Embryonic stem cells (ESCs) are derived from the inner cell mass of a blastocyst, which is formed 4–5 days after fertilization and exhibit the potential to self-renew without limit in culture. In more detail ESCs exhibit a high proliferation potential in vitro; maintain high levels of Oct-4 expression, telomerase activity, and a normal karyotype; and retain the potential to differentiate into cell types of all three lineages [1, 2].

Established human ESC lines were typically derived from in vitro fertilized embryos destined for destruction at in vitro fertilization units. In order to generate a single ESC line, 30–34 cells of the inner cell mass of blastocyst are isolated and expanded in vitro. Human ESC lines are cultured in growth medium supplemented with animal sera and maintained usually on mouse feeder layers (i.e., mouse embryonic fibroblasts) [3]. Furthermore, ESCs are pluripotent with a great differentiation potential to various cell types. The differentiation potential of human ESCs can be evaluated either in vivo or in vitro, whereas ESCs can be cultured in vitro under certain culture conditions to induce differentiation into the desired cell type [1, 4, 5]. The in vivo models involve injecting cells into immunocompromised mice and analyzing the teratoma formation. However, it is notable that established ES lines may display some genomic instability [5]. Thus, the use of ESCs for regenerative medicine is questioned, as ESCs appear to be tumorigenic and form teratomas that contain cell types representing all three primary germ layers in vivo [5]. It is evident that, prior clinical use, it will be important to exclude undifferentiated stem cells from cell types or products derived from ESCs. Another important issue that remains

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M. Gazouli, G. E. Theodoropoulos (eds.), *Digestive System Diseases*, Stem Cell Biology and Regenerative Medicine, https://doi.org/10.1007/978-3-030-11965-2_2

unsolved and must be addressed is the immune rejection that these cells may provoke. It has been generally assumed that due to the fact that human ESCs and their differentiated derivatives can express high levels of major histocompatibility complex (MHC) class I antigens, any ES cell-based product will be subjected to graft rejection [5].

Induced Pluripotent Stem Cells (iPSCs)

In 2006, Yamanaka et al. managed to reprogram mouse skin fibroblasts into stem cells, similar to embryonic stem cells, by overexpressing four transcription factors OCT4, SOX2, KLF4, and c-MYC; these cells were characterized as induced pluripotent stem cells (iPSCs) [6]. By using this approach, adult cells can be genetically reprogrammed to an embryonic stage by switching the expression of the necessary genes for the embryonic stem cell properties [6]. The iPSCs have the ability to further differentiate into various types of cells, like ESCs, without the presence of concomitant ethical problems associated with the destruction of the blastocyst. Accordingly, Yamanaka and et al. managed to reprogram the "biological clock of the cell." Since then, iPSCs have been generated from human somatic cells by using a variety of protocols. In subsequent studies, researchers replaced the original transcription factors with other combinations, but always in the presence of OCT-4, which represents an essential transcription factor for reprogramming somatic cells. The iPSCs resemble but are not identical to ESCs, as detailed genomic analysis reported the existence of epigenetic memory in iPSCs [7, 8].

To this end, iPSCs have been shown to possess some specific features or properties that can be acquired during the reprogramming process or are remnants of epigenetic modifications of the DNA derived from the parental tissue or cell that influence gene expression [7, 8]. These residual signatures of epigenomes and transcriptomes of the somatic tissue or cell of origin were termed as "epigenetic memory." It has been reported that residual DNA methylation signatures derived from the somatic tissue of origin may favor their differentiation potential into lineages related to the donor cell while restricting alternative cell fates [9].

The advantages and disadvantages of iPSCs can be summarized as follows:

Advantages: (i) iPSCs are undifferentiated with unlimited differentiation potential into all cell types. In addition, iPSCs can be expanded in vitro to a high passage. These properties are allowing them to be used as a potential therapeutic tool in all tissues and organs [8]. (ii) Studying iPSCs derived from pathological or normal tissue can offer a better understanding of a disease and the relevant molecular pathways. iPSCs are often termed as a "disease in a dish" [10]. (iii) No ethical considerations are related to iPS generation.

Disadvantages: (i) The efficiency or reprogramming is very often low and depends on the donor tissue and the reprogramming method. (ii) Prior transplantation into patients, it is needed to ensure that iPSCs are *fully differentiated* into the required

specialized cells. (iii) iPSCs, like ESCs, are reported to form teratomas in vivo after transplantation [7]. (iv) Epigenetic memory in iPSCs influences the gene expression [9].

Fetal Stem Cells (FSCs)

Fetal stem cells represent a relative new source of stem cells. These cells can be derived either from the fetus or from the supportive extraembryonic structures. FSCs have been recently isolated from several tissues such as amniotic fluid, amnion, umbilical cord blood, Wharton's jelly, placenta, fetal liver or fetal bone marrow [11–14].

Recent reports describe fetal stem cells as ideal cell types for regenerative medicine because they (i) are easily accessible as these cells are usually derived from tissues that are normally discarded following birth, such as umbilical cord, placenta, or amnion, (ii) exhibit high proliferation rates in vitro, (iii) do not form teratomas when injected to immunosuppressed mice in vivo, (iv) do not present ethical reservations like embryonic stem cells (ESCs) and (iiv) exhibit functional features indicating that they represent intermediates between ESCs and adult stem cells (e.g., amniotic fluid stem cells express the pluripotency marker Oct-4 in high levels [15, 16]). Another important issue is that early fetal stem cells appear to have pre-immune status and can be used with limited implications compared to adult stem cells in allogenic transplantations. In particular, these cells do not express HLA-class II, but express HLA-class I antigens, and they do not elicit lymphocyte proliferation in vitro [11–13].

However, these cells have a limited differentiation potential compared with ES cells, as they cannot give rise to all cell types of the three germ layers. It will remain necessary to show that fetal stem cells can differentiate into fully functional committed cells in vivo in order to evaluate better their therapeutic potential [11, 14].

In the following sections, fetal sources such as amniotic fluid, umbilical cord blood and extraembryonic tissues will be analyzed in detail.

Amniotic Fluid (AF)

AF serves as a protective liquid for the developing embryo, providing mechanical support and the required nutrients during embryogenesis. Amniocentesis has been used for many decades as a routine procedure for fetal karyotyping and prenatal diagnosis, allowing the detection of a variety of genetic diseases.

AF also represents a rich source of a stem cell population deriving either from the fetus or the surrounding amniotic membrane. Additional investigations by several groups have been recently focused on the cellular properties of amniotic-derived cells and their potential use in preclinical models and in transplantation therapies [12, 16–19].